I. Status of the Claims

Claims 1-7 are pending in the application and stand rejected. Claims 1 and 2 have been amended. New claim 8 has been added. No new matter has been added.

II. Amendments

Support for the claim amendments can be found throughout the application, including in the claims as originally filed. The limitation that the liposomes have number average size of about 50 to 300 nm can be found at page 18, ll. 19-20 of the specification, which specifies that about 50 to 300 nm is the preferred number average size of the liposomes.

The phrase "wherein the liposomes encapsulate the complexed bioactive agent" is supported throughout the specification. For example, support can be found at least in the abstract, which states that the invention is directed to methods of encapsulating complexes and to liposome encapsulated complexes. Support is also found in the preamble of claims 1 and 2, which recites "a method of encapsulating a bioactive complex in a liposome.

Support for deletion of the word "unilamellar" is found in claims as filed, which did not include this claim term as well as at page 17, ll. 19-28, which specifies that the liposome may be unilamellar or multilamellar.

The word "the" immediately preceding "water droplets" has been deleted in order to provide proper antecedent basis for "water droplets."

New claim 8 is supported by the specification at page 23, l. 22 to page. 24, l. 2. Specifically, condensed nucleic acids are described as "nucleic acids which have been combined with one or more polycations such that the nucleic acid strands are more tightly packed than would be the case in the absence of polycations."

No new matter has been added. Applicants respectfully request entry of the amendments.

III. Remarks

A. Rejection over Szoka in view of Gao or Papahadjopoulos

Claims 1-7 stand rejected under 35 U.S.C. § 103(a) as being allegedly obvious over *Proc. Natl. Acad. Sci. USA*, 1978, 75, 4194-4198 by Szoka et al. ("Szoka") in view of either U.S. Patent No. 5,795,587 to Gao et al. ("Gao") or in view of U.S. Patent No. 6,071,533 to Papahadjopoulos et al. ("Papahadjopolous"). Applicants respectfully traverse this rejection.

Applicants note that the present claims require the use of a complexing agent. The present claims further require that the complexing agent is added under particular conditions. Specifically, claim 1 recites combining a first aqueous suspension comprising a bioactive agent with the lipid containing organic solution to form an emulsion comprising the bioactive agent and the lipid, followed by adding a second aqueous suspension comprising a complexing agent to the emulsion. Claim 2 recites combining a first aqueous suspension comprising a complexing agent with the lipid containing organic solution to form an emulsion comprising the complexing agent and the lipid, followed by adding a second aqueous suspension comprising a bioactive agent to the emulsion. Both claims 1 and 2 require the step of incubating the emulsion to form a complex of the bioactive agent with the bioactive agent in lipid stabilized water droplets. Finally, claims 1 and 2 both require removing the organic solvent from the suspension to form liposomes comprising the complexed bioactive agent and the lipid, wherein the liposomes have an average size of about 50 to 300 nm, and wherein the liposomes encapsulate the complexed bioactive agent.

In order to establish a *prima facie* case of obviousness, a reference, or references when combined, must teach or suggest each and every claim limitation. Additionally, there must be some

suggestion or motivation, either in the references themselves or in the knowledge generally available to the skilled artisan, to modify a reference or combine reference teachings. Second, there must be a reasonable expectation of success. The teaching or suggestion must be found in prior art or knowledge of one of ordinary skill in the art, not in the applicant's disclosure. Finally, the reference, or references when combined, much teach or suggest each and every claim limitation. M.P.E.P. 2143.

Applicants submit that none of the cited reference combinations teach or suggest each and every limitation of the present claims. As explained below, neither Szoka, Gao, or Papahadjopoulos, either alone or in combination, teaches or suggests reacting a polycation and nucleic acid in a water in oil emulsion. Nor do any of these references teach or suggest forming a bioactive complex encapsulated in a liposome.

The Examiner relies on Szoka for teaching a method of preparing liposomes. The method "involves preparing a solution of a phospholipid in an organic solvent, mixing with an aqueous solution of the active agent to form an emulsion and evaporation of the solvent to form unilamellar liposomes," and that "this method is valuable for the encapsulation of RNA and DNA." Examiner acknowledges that Szoka does not teach "the addition of a complexing agent to the emulsion containing the active agent or addition of active agent to the complexing agent containing emulsion." (*Office Action* at p. 3.) To make up for Szoka's deficiency, Examiner relies on either Gao or Papahadjopoulos.

According to the Examiner, Gao teaches "liposomal delivery systems in which the nucleic acid is complexed with a polycation." (*Office Action* at p. 3.) Applicants respectfully submit that Gao fails to teach encapsulation of a bioactive active complex comprising a bioactive agent. Rather,

Gao forms a *lipid/DNA complex*, by mixing an aqueous buffer solution of DC-Chol/DOPE liposomes. (*Gao* at col. 12, ll. 23-35.) The resulting product is not an encapsulated DNA. (*Id.*) Similarly, Gao describes the preparation of a *lipid/DNA/polylactic acid complex* by mixing an aqueous buffer solution of DC-Chol/DOPE liposomes and polylactic acid with and aqueous buffer solution of DNA. (*Gao* at col. 12, ll. 23-35.) Again, this is not an encapsulated complexed bioactive agent. Gao further does not teach that the polycation and nucleic acid are reacted in a water in oil emulsion. This step, which results in the improved transfection efficiencies of the liposomes of the present invention, is completely lacking in either Szoka or Gao. Thus, the combined teachings of these references fails to render the claimed method obvious.

Papahadjopoulos is relied upon by the Examiner for teaching a "liposomal delivery system in which nucleic aid is complexed with a polycation such as spermidine, spermine and poly amino acids." Papahadjopoulos, however, also does not teach a method where the polycation and nucleic acid are reacted in a water in oil emulsion. Like Gao, Paphadjopolous describes the preparation of lipid/DNA complex by simply combining an aqueous lipid suspension with a an aqueous buffer mixture of plasmid DNA. Like Gao, no complexed bioactive agent encapsulated in a liposome is taught or suggested by Paphadjopolous. Thus, Szoka in combination with Papahadjopoulos also fails to teach each and every limitation of the instant claims.

The Examiner alleges that "[a]lthough Szoka does not teach the formation of the emulsion first with the complexing agent and then the addition of the active agent, it would have been obvious to one of ordinary skill in the art that a complex formation would occur whether the active agent is added to the complexing agent emulsion or complexing agent is added to the active agent containing emulsion since the complexation process is between an anionic agent and a cationic

agent." (*Office Action* at p. 4.) Nowhere does the Examiner point to any teaching or suggestion of forming an emulsion prior to forming the complexed bioactive agent. The Examiner merely states that it would have been obvious that such reagents would react because of their opposite charge.

Applicants respectfully disagree. One skilled in the art would not have a reasonable expectation of success in modifying the cited references in order to form an encapsulated bioactive complex. Rather, Gao and Papahadjopolous both teach and suggest that these steps would merely form a lipid-bioactive agent complex, such as an aggregate. Indeed, the uncontrolled complexation that occurs freely in aqueous solution, as in Gao and Papahadjopoulos results in aggregates too large to subsequently encapsulate with a lipid to form small liposomes. Applicants have amply demonstrated that small liposomes are produced by the claimed methods. The liposomes produced using the claimed method were characterized by size, as seen in Examples 5 to 7, p. 36, of the application. The micrographs of Figs. 7a to 7b also demonstrate the formation of small liposomes containing condensed nucleic acids. Applicants also direct the Examiner's attention to the attached reference by Shangguan et al. (Gene Therapy (2007) 7, 769-783), which also depicts better quality pictures of the same micrographs.

These references do not teach that the complexing agent will react with bioactive agent by way of exchange of these agents between the lipid stabilized water droplets, e.g., reverse micelles, as explained at page 27, ll. 22-27. This process is demonstrated by the cartoon at Figure 2 of the specification. Nor do these references provide the skilled artisan with any reasonable expectation of success that the specifically claimed steps will yield complexed bioactive agent encapsulated by a liposome.

Thus, the cited reference combination fails to teach or suggest each and every limitation of the claims. Accordingly, withdrawal of this rejection is respectfully requested.

B. Rejection over Szoka in view of Gao or Papahadjopoulos, in further view of Kim

The Examiner also rejects claims 1-7 as being allegedly obvious over Szoka in view of either Gao or Papahadjopoulos, in further view of U.S. Patent No. 5,723,147 to Kim et al. According to the Examiner, Kim discloses the preparation of liposomes in which the "lipid in an organic solvent is added with an aqueous solution of an active agent, which in turn is added, with a second aqueous solution containing a cationic amino acid lysine." (*Office Action* at p. 4.) The Examiner further alleges "[i]n essence, Kim teaches the addition of the active agent and the complexing agent by their introduction into the emulsion through two separate aqueous solutions." (*Id.* at p. 4-5.) Applicants respectfully traverse.

Applicants submit that lysine is not a bioactive complexing agent, much less a polycation as claimed. The Examiner points to no teachings or suggestions in any of the references or in the knowledge of one skilled in the art to support the assertion that lysine is a bioactive complexing agent. The method of Kim, is outlined at col. 3, ll. 38-57. Importantly, the method of Kim yields multivesicular liposomes having a diameter ± standard deviation of 19.4±6.5 microns. (*See also* Kim et al., attached.) Thus, the liposomes of Kim not only lack the complexing agent as claimed, but they are orders of magnitude larger than the liposomes produced by presently claimed methods, i.e. a number average size of 50 -300 nanometers.

Finally, the Examiner cites no motivation for combining any teachings of Kim with the teachings of Szoka, Gao or Hadjadopolous. Instead, the Examiner erroneously states that "[o]ne of

ordinary skill in the art would be motivated to add the active agent such as nucleic acid and the complexing agent through separate aqueous media to form a complex with a reasonable expectation of success since the reference of Kim shows it is routine practice in the art." (*Office Action* at p. 5.) As Applicants have repeatedly emphasized, lysine is not a complexing agent, and does not form a complexed bioactive agent as claimed. Applicants have defined a "bioactive complex" as "any bioactive agent bound to a complexing agent such that the complex thus formed results in a change in the physical properties such as decreasing the size of the bioactive molecule, decreasing the solubility of the bioactive agent, precipitating the bioactive agents, condensing the bioactive agent, or increasing the size of the complex." Thus, Kim neither teaches nor suggests adding a complexing agent and a nucleic acid through separate aqueous media to form a complex, as the Examiner contends. Thus, the cited reference fails to teach or suggest each and every limitation of the claims.

For at least these reasons, Applicants respectfully request withdrawal of this rejection.

IV. Conclusion

In light of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Reconsideration and timely allowance of the pending claims is respectfully solicited. If a telephone conference would be helpful, the Examiner is invited to call the undersigned at 617-832-1223. Please charge any additional fees required to enter this response, or credit any overpayment, to **Deposit Account No. 06-1448, TRA-016.01.**

Dated: September 7, 2007

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NONVIRAL TRANSFER TECHNOLOGY RESEARCH ARTICLE

A novel N-acyl phosphatidylethanolamine-containing delivery vehicle for spermine-condensed plasmid DNA

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A unique method for formulation of plasmid DNA with phospholipids has been devised for the purpose of producing vehicles that can mediate gene delivery and transfection of living cells. The polycation, spermine, was used to condense plasmid DNA within a water-in-chloroform emulsion stabilized by phospholipids. After organic solvent removal, the particles formed could be extruded to a number average size of about 200 nm and retained DNA that was protected from nuclease digestion. This resulted in a relatively high protected DNA-to-lipid ratio of approximately 1 μg DNA/μmol lipid. The size distribution of the preparation was relatively homogeneous as judged by light microscopy and quasi-elastic light scattering. Electron microscopic studies showed structural heterogeneity, but suggested that at least some of the plasmid DNA in this preparation was in the form of the previously observed spermine-condensed bent rods and toroids and was encapsulated within liposomal membranes. Preparations with the fusogenic phospholipid composition, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-dodecanoyl/1,2-dioleoyl-sn-glycero-3-phosphocholine, showed transfection activity for several cells lines, particularly OVCAR-3 cells. The transfection activity sedimented with the lipid during centrifugation, confirming the association of active plasmid DNA with phospholipids. Transfection efficiency in culture was found to be of the same order of magnitude as cationic lipoplexes but much less toxic to the cells. Significant transfection of OVCAR-3 cells in tissue culture could also be observed, even in the presence of the intraperitoneal fluid from a mouse with an OVCAR-3 ascites tumor. These data indicate a new type of liposomal gene delivery system devoid of cationic lipids, phosphatidylethanolamine, cationic polymers and viral components. Gene Therapy (2000) **7**, 769–783.

Keywords: *liposome; gene delivery; anionic; spermine; N-acyl-phosphatidylethanolamine*

Introduction

In a relatively short period of time, gene therapy has progressed from basic research to clinical trials.[1–3] Two major groups of delivery systems are being developed for this purpose, viral and nonviral. Although quite efficient, the use of various viral systems[4] has been hampered by toxicity, the immunogenicity of viral components,[5] potential risk of reversion to a replication-competent state, potential introduction of tumorigenic mutations, lack of targeting mechanism, limitations in DNA capacity and difficulty in large-scale production.[6]

Nonviral delivery systems such as cationic liposome–DNA complexes (or lipoplexes)[7] and polymer complexes (or polyplexes)[8,9] have also been developed to circumvent some of these problems. Typically, such complexes protect the DNA from extracellular nucleases by condensation. Despite their advantages, many lipoplexes and polyplexes suffer from certain drawbacks.[6]

Plasmid DNA must move through a series of different environments requiring different properties of a delivery system before ultimate expression occurs in the nucleus of the target cell. In particular, the requirement for most types of lipoplexes and polyplexes to have an overall positive charge leads to nonspecific association with negatively charged cell membranes. Furthermore, *in vivo* stability of these exposed ionic complexes limits condensing agents to those with very strong interactions. On the other hand, the apparent necessity for the DNA complex to easily dissociate after delivery into the cytoplasm to permeate nuclear pores[10–13] suggests that a delicate balance between condensation and dissociation must be found. To allow the greatest flexibility in choosing a DNA condensing agent, it would be preferable to limit its exposure to the physiological millieu until the condensed DNA has entered the cell.

One way in which this can be done is by physically separating the DNA from the extracellular environment before cytoplasmic delivery. This can potentially be accomplished using another category of nonviral vectors, true liposomes, which encapsulate plasmid DNA within an encompassing bilayer. The DNA in such a structure would be well protected from nucleases, allowing the use of any condensing agents as well as the coencapsulation of transgene activators or nuclear localization signals. Furthermore, the separation of condensing and structural functions allows great flexibility in the characterisitics of the surface of the liposomal particles, eg in terms of charge or hydrophilicity.

A strong rationale for condensation of the DNA inside of liposomes continues to exist because of the importance of particle size. An ideal liposomal vector should be

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smaller than 400 nm in size to avoid rapid clearance *in vivo*.[14] Small liposomes also tend to be more fusogenic than large liposomes[15,16] and thus may provide higher transfection efficiency. Furthermore, smaller liposomes may undergo endocytosis more readily and avoid nonspecific binding better than larger particles. In addition, smaller liposomes are more often unilamellar, an important prerequisite for efficient fusogenic delivery into the cytoplasm. However, the large extended size of plasmid DNA makes it difficult to pack into small liposomes. Polycations of charge +3 or higher, such as polylysine,[17] polyamines[18–20] and hexammine cobalt (III),[20,21] have been reported, *in vitro*, to condense plasmid DNA into much smaller toroid or rod-shaped structures through charge neutralization.[22]

Polyamines, such as spermine, can dissociate from DNA at cytoplasmic salt concentrations, potentially releasing the DNA for transport into the nucleus.[18,19,22] Unlike other polycations or cationic lipids, the polyamines, spermidine(3+) and spermine(4+), occur naturally in all living cells.[23,24] Millimolar concentrations of polyamines have been found in viruses and bacteria and are involved in packaging of viral and bacterial DNA.[23,25] Such high levels of polyamines also exist in actively proliferating animal cells[23,24] and are essential for maintaining normal cell growth.[26,27] Polyamines have been found to enhance the rates of replication, transcription and translation.[28–31] DNA condensed *in vitro* with spermidine has shown higher transcriptional activity than uncondensed DNA.[32] Therefore, the polyamines may constitute one particularly useful class of condensing agents for liposomal delivery systems.

Liposome composition is flexible when the lipids are not required to condense DNA. Liposomes may be composed of lipids of anionic or neutral charge and may contain small proportions of cationic lipids. Since most naturally occurring lipids are either neutral or anionic, liposomes are less likely to be toxic.[6] Because they form true bilayers around encapsulated DNA, the exposed surface properties of each particle can be tailored to satisfy the appropriate physiological constraints independently of the DNA condensation system. Moreover, incorporation of fusogenic lipids and targeting molecules into the membranes of such liposomes may be used to increase the efficiency and the specificity of the delivery by enhancing fusion with the desired cell membranes.

While many types of fusogenic lipids have been identified, the anionic *N*-acyl phosphatidylethanolamines are one of the few shown to mediate direct delivery of internal liposomal contents through real cell membranes, ie red cell ghosts, apparently by a fusogenic mechanism.[33] In this study, we present a stage in the development of a new approach to formulation of condensed plasmid DNA with liposomal phospholipid membranes, including characterization of the products and demonstration of the formation of a unique anionic transfection-active vehicle based on the *N*-acyl-phosphatidylethanolamines. These differ from most nonviral systems in the lack of cationic lipids, phosphatidylethanolamine, cationic polymers and viral components.

Results

Direct condensation of DNA with spermine

Our goal was to encapsulate plasmid DNA into fusogenic liposomes for delivery into living cells. Optimal encapsulation of plasmid DNA into liposomes would be expected to be promoted by using the highest lipid concentration possible so that most of the aqueous solution can be entrapped within the liposomes. The DNA concentration consequently must also be very high to ensure that the maximum possible percentage of the liposomes are loaded with plasmid DNA. Conditions were chosen to try to meet these criteria. In particular, we based our initial conditions (30 μmol of phospholipid, 250 μl of buffer, 200 μg of pZeoLacZ plasmid DNA) on the assumption of 200 nm spherical liposomes. The aqueous volume and DNA concentration were chosen to allow most of the volume to be encapsulated and at least one plasmid molecule per 200 nm liposome.

Condensation of individual plasmids can be achieved using polyamines such as spermine and spermidine[18,19] at very diluted plasmid concentrations to avoid aggregation. However, the resulting DNA concentration in our preparations, as described above, would be much higher than those previously used to demonstrate polyamine condensation. It was expected that aggregation would be a major factor under such optimal loading conditions. None the less, we first tested the precondensation of the DNA for potential encapsulation into liposomes. Indeed, when equal volumes of the DNA (200 μg pZeoSVLacZ plasmid DNA, 6.5 kb, in 125 μl low salt buffer, designated LSB, see Materials and methods) and spermine aqueous solutions (7 mM spermine in 125 μl LSB) were mixed gently, the expected massive aggregation occurred as judged by a large turbidity change of the solution. Microscopic observation (Figure 1a) demonstrated that the aggregates were generally much larger than 1 μm and often as large as 5–10 μm. The large size of these aggregates was further confirmed by cryo-electron microscopy (Figure 1b). Of particular note at this magnification are the regular arrays of fibers, perhaps as a result of spermine-induced condensation to a partially ordered structure. There were also some curved rods suggestive of the beginnings of toroidal structures, but no complete toroids. Obviously, the observed aggregates were too large to be useful for a delivery system. It seemed unlikely that relatively small particles in the range of a few hundred nm could be easily prepared by this method. Polycation-induced aggregation is likely to be a general problem for DNA condensation and encapsulation into liposomes under these conditions.

Two-step condensation method

Since the direct condensation with spermine at high plasmid concentration yielded aggregates even larger than the original 0.5–1 μm average length of the free plasmid,[32,34] it was necessary to find another way to condense plasmid DNA for potential encapsulation into liposomes. In the commonly used water-in-oil emulsion methods for liposomal preparation,[35,36] phospholipids stabilize reverse micelles before solvent is removed. Reverse micelles have long been used as a reaction medium to mimic the microenvironment that enzymes encounter inside cells.[37] In these systems, the size of individual water droplets is determined largely by the amphiphile-to-water ratio. The droplets are sequestered from each other by the solvent and the only communication between two water droplets is through the formation of transient reverse micelle dimers.[38] Intermicellar solute exchange can occur in these transient dimers.[39] However,

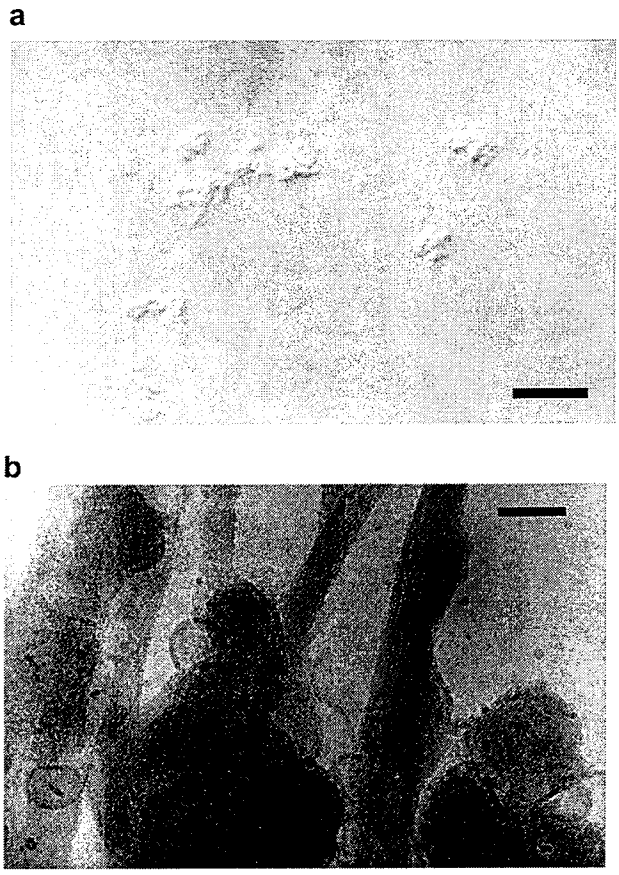

Figure 1 Micrographs of spermine-mediated plasmid DNA aggregation. Two hundred μg plasmid DNA in 125 μl LSB was mixed gently with 7 mM spermine in 125 μl LSB. (a) Light microscope observation after 15 min incubation at room temperature. Bar represents 10 μm. (b) Cryo-TEM observation. Bar represents 100 nm.

the rate of exchange of large molecules, such as proteins, is dramatically slower than the rate of exchange of small molecules and salts.

Based on these observations, we hypothesized that spermine-mediated DNA aggregation in concentrated DNA solutions might be inhibited if the condensation were performed in reverse micelles stabilized by the lipids used to make the liposomes. Our strategy was to first form a sonicated water-in-solvent emulsion with plasmid followed by addition of aqueous spermine solution to this emulsion and further sonication, ie a two-step process (Figure 2). The phospholipid-to-water-ratio was chosen based on the expected surface area to be occupied by each lipid molecule so that initial reverse micelles would be relatively small. Because of the large size and the surface charges of the plasmid and the particular phospholipid-to-water ratio, it would be likely that each reverse micelle would contain a single or small number of plasmid molecules. It would also be unlikely for plasmids to exchange rapidly between micelles. However, we expected spermine to be exchanged quite rapidly into the plasmid-containing droplets, leading to condensation of the plasmid. Thus, we hypothesized that DNA aggregation would be inhibited, allowing stable condensation of individual or small groups of plasmids until the

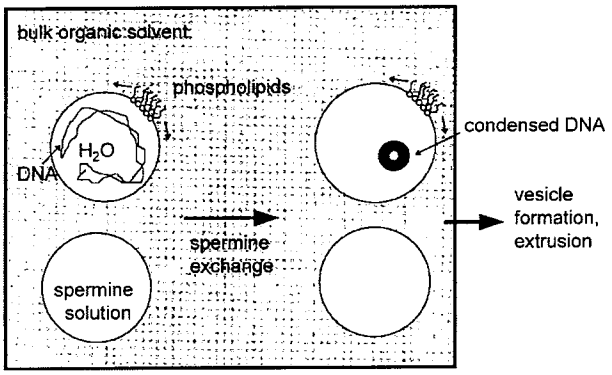

Figure 2 Schematic representation of two-step emulsion method of DNA encapsulation. Condensation of DNA occurs within phospholipid-stabilized water droplets that have formed around the DNA in a bulk organic solvent. Separate spermine-cotaining droplets transfer spermine into the DNA containing droplets by transient contact and exchange. After condensation within the emulsion, vesicles are formed by solvent evaporation and further extruded to smaller sizes.

liposomes could be formed around the DNA by solvent evaporation.

Plasmid DNA formulation with fusogenic lipids

Since our goal was to deliver plasmid DNA into cells via membrane fusion, we chose to test a novel fusogenic liposome formulation, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-dodecanoyl/1,2-dioleoyl-*sn*-glycero-3-phosphocholine (*N*-C12-DOPE/DOPC) (70:30), previously shown to fuse with and deliver encapsulated material into erythrocyte ghosts[33] and nucleated cells (Ahl *et al*, unpublished data) in a cation-dependent manner. *N*-C12-DOPE bears a net negative charge which could potentially interact with positively charged spermine and affect the condensation process. Therefore, it was necessary to test the spermine partitioning between DNA and liposomes of this composition in a low salt buffer dialysis experiment. A three-chamber dialysis device was used as described in Materials and methods to monitor the ability of the liposomes to sequester spermine from DNA. Spermine-mediated DNA aggregation was monitored at various spermine concentrations allowing construction of spermine–DNA binding curves and showing that the presence of the liposomes only slightly shifts the curve for spermine binding to DNA in this case (data not shown). Spermine had an apparent binding constant for DNA 178 times higher than for the acidic phospholipids. The predetermined optimal concentrations of spermine were well above the amounts necessary to condense the DNA in all preparations.

Two *N*-C12-DOPE/DOPC (70:30) samples and control egg phosphatidylcholine (PC) samples were then prepared using the pZeoSVLacZ plasmid in the two-step procedure described above. Solvent was removed from the emulsions and the preparations were initially suspended in a hyperosmotic sucrose solution to minimize any potential leakage while they were extruded through 0.4 μm pore size membranes. After preparation each sample was extensively dialyzed into a physiological salt buffer to remove unencapsulated spermine and uncondense any DNA that was not encapsulated.

Protection of plasmid DNA from DNase digestion was

then evaluated by agarose gel electrophoresis (Figure 3) for a sample prepared with spermine and a control sample prepared without spermine (to compare transfection activity later). No additional bands were visible in the gel as a result of the preparation procedures, indicating intact plasmid DNA. To compare the encapsulation efficiency of the two methods, aliquots of each preparation were digested with DNase I and compared with samples not digested. Both preparations allowed significant DNA protection or apparent encapsulation. To quantify this protection, DNA was extracted from each aliquot and measured by the Picogreen fluorescent assay (see Materials and methods). Under our experimental conditions, the fluorescent signal from digested DNA was insignificant (data not shown). The sample with spermine displayed 10.1 ± 5.6 ($n = 3$) percent plasmid protection, while 19.0 ± 4.5 ($n = 3$) percent was protected in the sample without spermine. In terms of the encapsulated DNA/lipid ratio, a minimum value of 0.80 ± 0.16 ($n = 4$) $\mu g/\mu mol$ was obtained by measuring the protected DNA and assuming that all of the lipid had been recovered. The amounts of various components of the initial emulsions can be varied to increase this ratio potentially.

Particle size analysis

The *N*-C12-DOPE/DOPC preparations were then characterized by quasi-elastic light scattering using a NICOMP submicron particle analyzer to determine whether they were of the size expected for liposomes prepared in this manner. The samples with and without spermine could be fit by Gaussian size distributions with number average particle sizes of 222.6 nm (±94.2 nm at half height) and 141.2 nm (±60.8 nm at half height), respectively. An egg PC sample prepared with spermine was 155 nm (±71 nm at half height). The number averages reflect the expected predominant number of liposomes in the Gaussian fit that are significantly smaller than the pore size. The total number of liposomes for the *N*-C12-DOPE/DOPC samples can be estimated from the Gaussian summary mode where the total distribution is divided into 22 bins and each bin is taken as a homogenous population of the average size and in the relative abundance indicated by the analyzer software. At the 30 μmol lipid scale the total number of unilamellar liposomes is estimated to be 3×10^{13} for the spermine-condensed sample based on the measured liposomal size distribution. If some liposomes were multilamellar, the number of liposomes would have been smaller. The total number of encapsulated DNA molecules is 6×10^{12}, indicating that it is possible that more than 20% of the liposomes could have encapsulated individual plasmid DNA molecules.

Further confirmation of the particle size was obtained from light microscopy, comparing these samples with polystyrene beads with an average diameter of 269 ± 7 nm. Figure 4 shows the photomicrographs of the sample made with spermine by the two-step method (a) and the beads (b). The sample particles appeared relatively uniform in size and shape at this magnification, and the approximate size of sample particles appeared similar to those obtained from our dynamic light scattering studies. Of particular note is comparison of this sample with the spermine-aggregated DNA in Figure 1a. There is no evidence of the large aggregates observed when spermine interacts directly with DNA in aqueous solution, indicat-

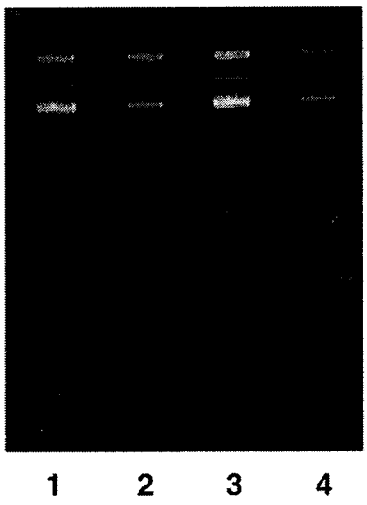

Figure 3 Agarose gel analysis of plasmid DNA protection in N-C12-DOPE/DOPC (70:30) formulations. An aliquot from each preparation after extrusion and dialysis was divided and one part digested with DNase I (see Materials and methods). The DNA in each aliquot was then extracted with phenol/$CHCl_3$, precipitated with ethanol and run on a 0.8% agarose gel. Lane 1, two-step preparation without spermine; lane 2, same as lane 1 but digested with DNase I; lane 3, two-step preparation with spermine; lane 4, same as lane 3, but digested with DNase I.

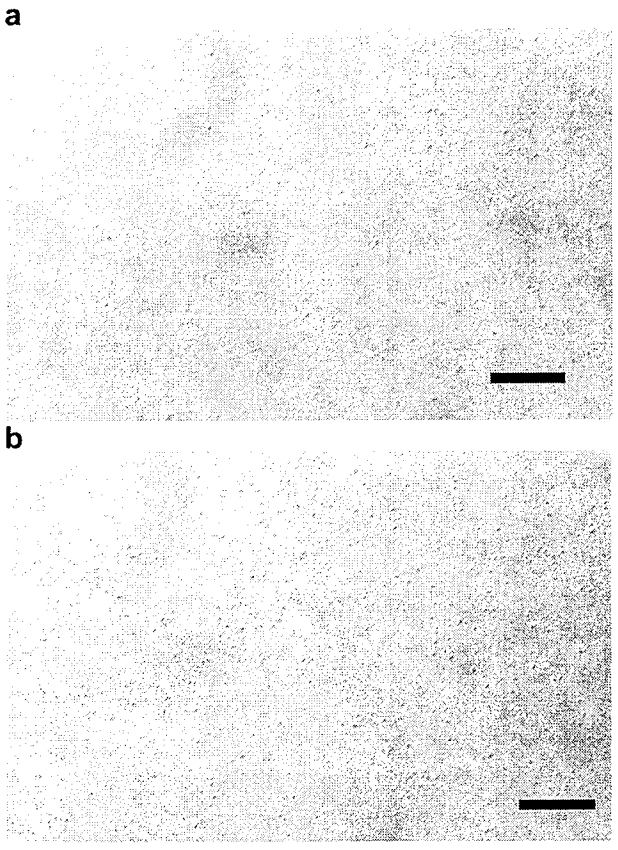

Figure 4 Light micrographs of the particles in N-C12-DOPE/DOPC (70:30) sample prepared as described by the two-step method with pZeoLacZ plasmid and spermine (a) versus polystyrene beads with an average diameter of 269 ± 7 nm (b). Bars represent 10 μm.

ing that the emulsion condensation method may greatly inhibit such aggregate formation and/or allows some of such aggregates to be broken up by the subsequent extrusion process.

Ultrastructural analysis

These potentially fusogenic preparations were then further characterized by freeze–fracture transmission electron microscopy (TEM). The preparation without spermine (data not shown) and most of the particles in the preparation with spermine (Figure 5) had the appearance of normal liposomes. Most of the particles were small in size, consistent with NICOMP results. Because of the prevalent fracture plane through lipid bilayers, observation of internal contents is rare with this technique. However, a small number of the particles seemed to have some encapsulated structures, which could represent condensed DNA (Figure 5, arrow).

Cryo-EM was then used to ascertain the liposomal nature of the preparations and possibly to visualize any encapsulated materials. When spermine was omitted from the procedure for the *N*-C12-DOPE/DOPC liposomes, primarily unilamellar, relatively small but structurally heterogenous liposomes were observed (Figure 6a), consistent with the NICOMP analysis. A number of liposomes appeared to be tubular, probably as a result of the osmotic gradient generated during the preparation procedure. Some liposomes showed interior fiber-like structures possibly representative of uncondensed DNA (arrow). Unencapsulated free fibers could also be seen (star).

Samples prepared with spermine (Figure 6b, c) were also somewhat heterogeneous in size, shape and lamellarity. Some particles were normal-looking liposomes with no visible encapsulated material. However, others contained electronically dense, well-defined toroidal structures (Figure 6b, arrow) that were not seen in the samples without spermine. Such structures were not related to the particular lipid used, as toroidal (Figure 6c, arrow) and bent rod structures (Figure 6c, pound sign) were observed in egg phosphatidylcholine (PC) preparations, which tended to be more stable under the cryo-EM sample preparation conditions. The spacing between the fine lines within the rods and toroids were uniform and significantly smaller than the spacing between two membranes in multilamellar liposomes (star). These toroidal structures bear great resemblance to the toroids and rods observed when free DNA is condensed by spermine[18] or other condensing agents[19,21,40] in dilute solutions. The parallel and concentric fine lines visible within the rods and toroids also resembled the lines seen within the plasmid aggregates (Figure 1b). Although there is no

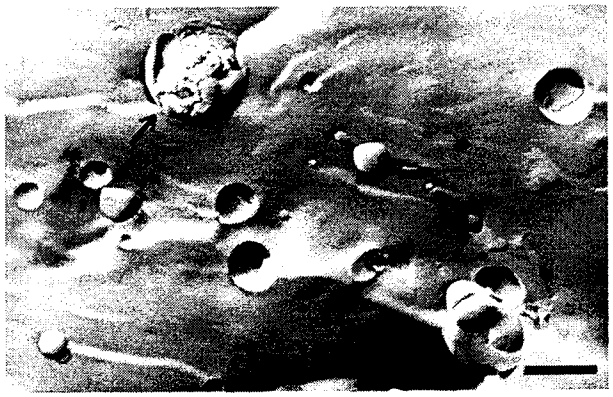

Figure 5 Freeze–fracture TEM micrographs of N-C12-DOPE/DOPC (70:30) samples prepared by the two-step method with plasmid and spermine. Arrow points to the particle with apparently encapsulated material. Bar represents 400 nm.

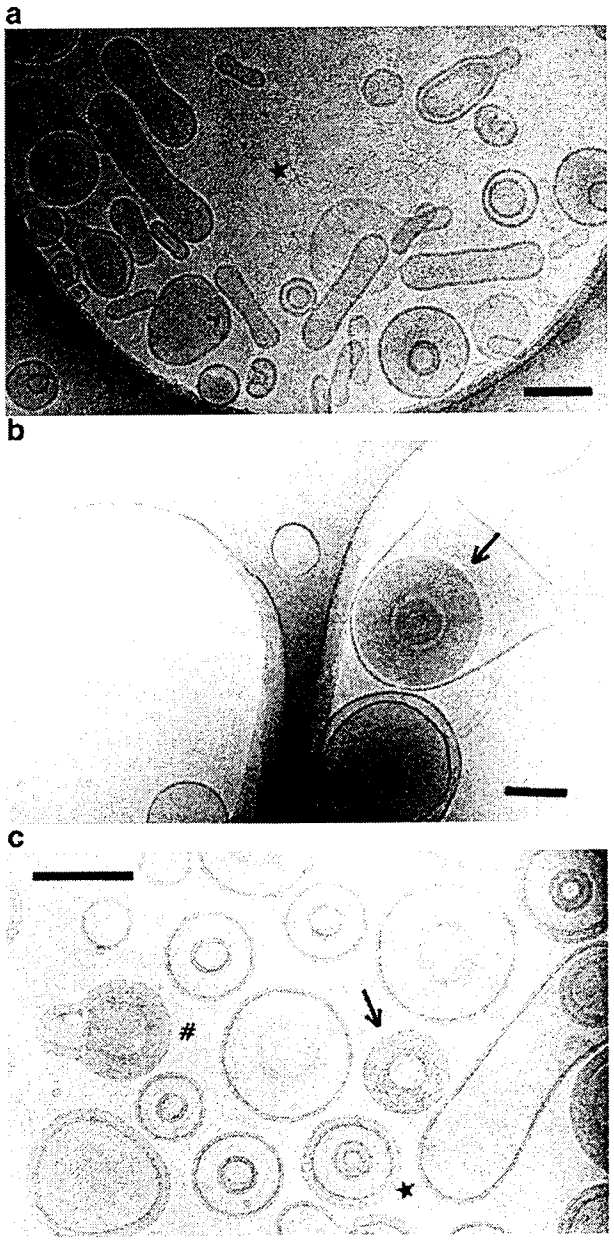

Figure 6 Cryo-TEM micrographs of two-step preparations with N-C12DOPE/DOPC and the pZeoLacZ plasmid without spermine (a) or with spermine (b). Markers in (a) designate fiber-like structures outside (*) and apparently inside (→) liposomes. In (b), an arrow points to a toroid that resembles polycation-condensed plasmid DNA. Bars in (a) and (b) represent 100 nm. Photomicrograph (c) represents an EPC sample made with the two-step procedure with spermine. Toroid (→) and bent rod (#) structures are compared with multilamellar liposomes (*). Bar represents 50 nm.

definitive identification of any particular structure as DNA in these micrographs, it is likely that at least some of the observed internal structures represent condensed plasmid DNA.

Membranes could be clearly observed around some of the toroidal structures (eg Figure 6b). It is likely that all of the observed toroids are encapsulated within an ion-impermeable barrier, since condensed DNA toroids cannot exist in the high salt buffer in which the liposomes were ultimately suspended. Therefore, it would appear that a major proportion of these preparations consists of liposomally encapsulated plasmid DNA. One unique structure found occasionally in the spermine-condensed *N*-C12-DOPE/DOPC samples and not in egg PC samples was large and honeycomb-like (data not shown) and may represent cubic phase lipid. Since neither the honeycomb structure nor the large liposomes were observed by freeze–fracture TEM, it is possible that they are simply artifacts resulting from the cryo-TEM preparation techniques. However, the tendency to form such structures could relate to the fusogenic nature of these membranes.

Characterization of transfection activity; role of spermine

The ultimate goal of formulating plasmid DNA with *N*-C12-DOPE/DOPC (70:30) was to deliver the plasmid DNA into cells with subsequent expression of the delivered gene. Initial studies showed that these lipids formulated with the pZeoSVLacZ plasmid could be used to transfect confluent OVCAR-3 cells with expression of β-galactosidase (data not shown). We later found that transfection could be demonstrated with higher sensitivity using the pEGFP-C1 plasmid which expresses an enhanced green fluorescent protein. Figure 7 shows cells incubated with pEGFP-C1-containing samples in serum-free RPMI 1640 medium. Substantial transfection of a significant percentage of the cells (estimated by microscopic observation to be in the range of 1–10%) was observed without further optimization of this system (Figure 7a). It is also clear from these data that even though more DNA appears to be encapsulated in the absence of spermine, the transfection efficiency of the samples without spermine (Figure 7b) is essentially undetectable, as opposed to the formulation with spermine (Figure 7a). Quantitative measurement of transfection (Figure 8) also showed that only the spermine-containing preparation (Figure 8a *versus* b) had significant activity. Therefore, this condensing agent is necessary for efficient gene expression.

An important control was to test the transfection activity of the appropriate amount of free DNA with empty liposomes, since external DNA had not been removed from these initial preparations. As shown in Figures 7c and 8c, no transfection activity could be detected under these conditions, indicating that it is the protected, apparently encapsulated, presumably condensed plasmid that is responsible for transfection rather than the considerable amount of free and/or unprotected plasmid DNA in these preparations (see above, Figure 3).

Role of phospholipids in transfection activity

The association of the transfection activity with fusogenic lipids was tested by comparison with an egg PC preparation (Figure 8d). Despite the fact that structurally similar vesicles with similar encapsulation efficiency were observed (data not shown and Figure 6c), there was no

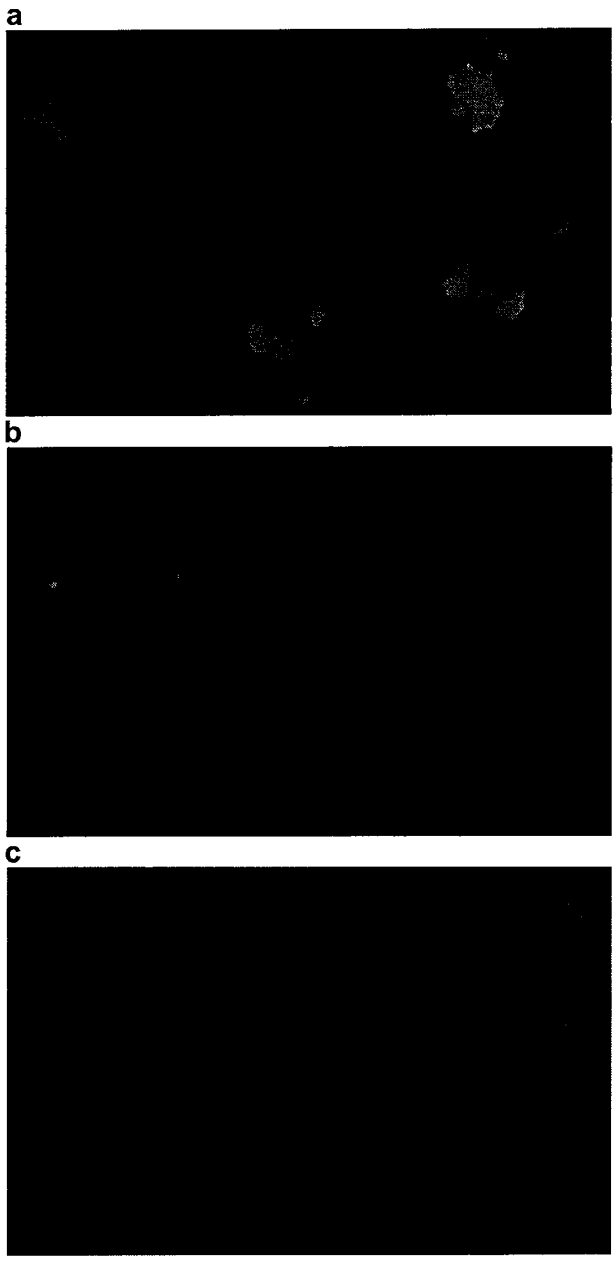

Figure 7 *Fluorescence photomicrographs of confluent OVCAR-3 cells after transfection with the N-C12-DOPE/DOPC (70:30) preparations. Confluent cells were incubated with the samples in serum-free medium for 3 h. After the samples were removed, the cells were incubated for 22 h in medium containing 10% fetal bovine serum and trichostatin. The cells were washed with PBS before being photographed. Liposomal samples were prepared with pEGFP-C1 plasmid DNA by the two-step method (a) with spermine or (b) without spermine (final lipid concentration approximately 2 mM, final total DNA concentration approximately 2 μg/ml encapsulated, 14 μg/ml including external DNA). A sample (c) of empty N-C12-DOPE/DOPC (70:30) liposomes without spermine plus free pEGFP-C1 plasmid DNA added outside the preformed liposomes was also tested. The amount of plasmid DNA added to the empty liposomes in sample c was equal to the total amount in each of the other preparations. Equal liposome concentrations were used in the experiments.*

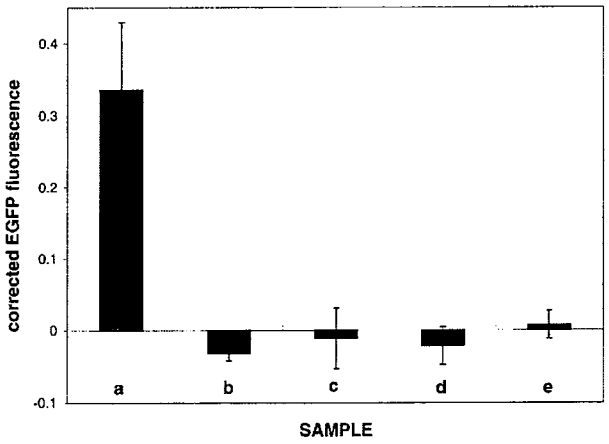

Figure 8 Quantification of EGFP expression in OVCAR 3 cells transfected with pEGFP-C1, as measured by the EGFP fluorescence level. Transfection experiments (a, b and c) were the same as in the previous figure caption. In addition we tested (d) egg PC liposomes prepared with spermine and pEGFP-C1 plasmid by the two-step method and (e) no additions. The cells were washed and labeled with calcein blue acetoxy methyl ester (CBAM) as described in Materials and methods and then dissolved in detergent to measure the fluorescence of EGFP and calcein blue as described. Fluorescence is expressed as a ratio of EGFP and calcein blue fluorescence, ie normalized for total cellular esterase activity (see Materials and methods). Estimated equivalency in units per cellular protein is given in Materials and methods. Error bars are ± s.d.

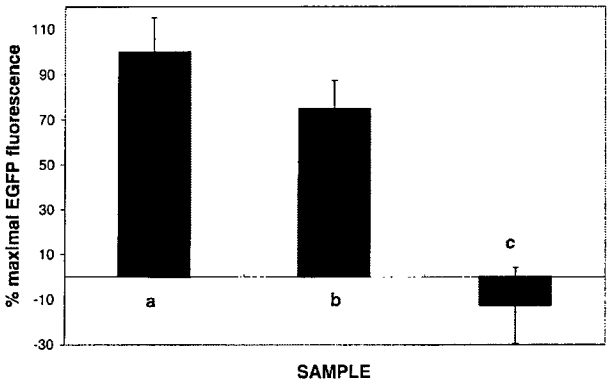

Figure 9 Association of transfection activity with the lipid pellet of N-C12-DOPE/DOPC (70:30) prepared with spermine and pEGFP-C1 plasmid DNA by the two-step method. A sample was prepared as before, but the intial plasmid DNA and spermine solutions contained 200 mM sucrose. After extrusion and dialysis, half of the sample was used for transfection without further handling (a), and the lipid particles from the rest of the sample were pelleted by centrifugation and washed once with HBSS before being used for transfection (b). An N-C12-DOPE/DOPC (70:30) sample with only the 200 mM sucrose was also prepared, and plasmid DNA and spermine were both added externally just before dialysis at an amount equal to that used in the other samples. The pellet of this empty sample (c) was prepared the same way as described above. Then, an equal lipid amount (approximately 2 mM) of each of the samples was used for transfection under the conditions described in the previous figure captions (see Materials and methods). After overnight incubation, the cells were labeled with CBAM and the fluorescence of EGFP and calcein blue were measured as described in Materials and methods. Estimated equivalency in units per cellular protein is given in Materials and methods. Error bars are ± s.d.

transfection activity with egg PC liposomes containing spermine-condensed plasmid. Therefore, the transfection activity would appear to be associated with the presence of the membrane-active lipid, *N*-C12-DOPE.

It was possible at this point to demonstrate further that transfection activity is associated with fusogenic lipids by a separation of the *N*-C12-DOPE/DOPC (70:30) lipid particles from free DNA when 200 mM sucrose was incorporated into the liposomal preparation procedure. The dense encapsulated sucrose allowed a mild centrifugation followed by a wash to pellet approximately 80% of the lipid under these conditions, while only about 14% of the total DNA pelleted. Without coencapsulation of sucrose, a partially soft diffuse pellet resulted with only about 7% of the DNA in the tight part of the pellet, suggesting that an aqueous space for sucrose encapsulation is associated with at least some of the DNA.

The transfection activity of the pellet was then tested. It was found to be comparable with that of the whole sample (Figure 9a and b), further indicating that the transfection activity is associated with lipid. As a control, free plasmid DNA was mixed with spermine and sucrose-containing empty *N*-C12-DOPE/DOPC (70:30) liposomes. These were dialyzed against Hanks buffer as in the original protocol and also pelleted. About 80% of the lipid was found in the pellet again, while the amount of plasmid DNA in the pellet was negligible. Therefore, free plasmid DNA does not pellet under these conditions. Furthermore, there must be no significant spermine-mediated association between plasmid DNA and the empty liposomes under these conditions. This pelleted material also showed no transfection activity (Figure 9c). Since free plasmid DNA does not pellet under the conditions of our experiments and transfection activity is associated with a lipid fraction that apparently encapsulates sucrose, it is strongly suggested that transfection activity is associated with plasmid DNA encapsulated in true liposomes.

Further support for the association of the transfection activity with fusogenic liposomes came from the subsequent observation that the transfection activity of the *N*-C12-DOPE/DOPC (70:30) liposomes could be enhanced up to 10-fold when the Ca^{2+} and Mg^{2+} levels of the RPMI 1640 medium were increased to fall within the normal range for blood serum (1.2 mM Ca^{2+}, 0.8 mM Mg^{2+}; data not shown). At higher Ca^{2+} concentration (2 mM) up to 50% of the cells in a well appeared to be transfected by fluorescence microscopic observation (data not shown). The cation-dependent fusogenicity of liposomes of this lipid composition (without DNA) had been previously established.[33] In addition, the transfection activity of the pelleted and washed liposomes was again in the same range as the whole sample even when physiological Ca^{2+} and Mg^{2+} levels were used during transfection (in two experiments, the pellet activity was 110 ± 20% and 95 ± 26% of the whole sample).

Comparison with cationic lipoplexes

The pelleted liposomes free of external DNA could also be used for direct comparison of transfection with cationic lipoplexes at equal DNA concentrations. These data are presented in Table 1 relative to the liposomal treatment (all data after incubation in sodium butyrate, a non-toxic activator of transgene expression[41–43] and at physiological Ca^{2+} and Mg^{2+} levels; all data normalized in terms of total intracellular esterase activity). In the table, the cell viability and transfection are taken as 1.0 for the *N*-C12-DOPE/DOPC liposomes, ie numbers greater than 1.0

Table 1 Cell survival and transfection efficiency of cationic lipid complexes relative to *N*-acyl-PE-containing liposomes (defined as 1.0)[a]

Lipid/ Mixture[b]	*Relative cell survival 2 μg/ml DNA*[c]	*s.d.*	*Relative transfection 2 μg/ml DNA*[c,d]	*s.d.*	*Relative cell survival 10 μg/ml DNA*[c]	*s.d.*	*Relative transfection 10 μg/ml DNA*[c,d]	*s.d.*
1	0.631	0.078	0.619	0.203	0.313	0.011	0.942	0.222
2	0.651	0.084	0.987	0.216	0.401	0.009	0.794	0.207
3	0.639	0.059	0.833	0.272	0.352	0.006	1.186	0.267
4	0.739	0.078	1.655	0.382	0.297	0.017	1.327	0.395
5	0.618	0.070	0.096	0.064	0.460	0.011	0.091	0.024
6	0.704	0.077	1.050	0.224	0.366	0.008	1.182	0.266
7	0.660	0.069	0.096	0.030	0.431	0.008	0.802	0.231
8 (Lipofectin)	0.708	0.089	1.651	0.381	0.139	0.014	0.325	0.077
9 (DC-chol/ DOPE)	1.095	0.101	4.930	0.991	0.383	0.032	2.451	0.627

[a]Ovcar-3 cells were incubated with washed liposomal pellets (as in Figure 9b) or lipid complexes with equal amounts of pEGFP-C1 plasmid DNA for 3 h in serum-free medium. All transfection procedures were as described in Materials and methods and include adjustment of Ca^{2+} and Mg^{2+} levels to 1.2 and 0.8 mM, respectively.
[b]Cationic lipid complexes were prepared with the following lipids: No. 1, 1:1 mixture of Tris-((2-glutaroyl-4-amino-*N*-dioctadecyl amine)-4′-(2,5-diaminopentanoyl-(2″,5″-diaminopropylethyl)) amine, trifluoroacetate and 2-amino-(2′,2′-dimethyl)ethyl-methylphosphonic acid-*O*-octadecyl-(1′-heptadecyl) ester, trifluoroacetate (Pfx-1); No. 2, 2,5-diaminopentanoyl-glycyl-glycyl-*N*-octadecyl-(1′-heptadecyl) amide, trifluoroacetate (Pfx-2); No. 3, 1:1 mixture of 2,5-diaminopentanoyl-(2′,3′-di-3-aminopropyl)-2-aminoacetyl-2-aminoacetyl-*N*-octadecyl-(1′-heptadecyl) amide, trifluoroacetate and DOPE (Pfx-3); No. 4, 1:1 mixture of 2-amino-(2′,2′-dimethyl)ethyl-methylphosphonic acid-*O*-octadecyl-(1′-heptadecyl) ester, trifluoroacetate and 2,5-diaminopentanoyl-2-aminoacetyl-*N*-dioctadecyl amide, trifluoride (Pfx-4); No. 5, 1:1 mixture of 2,5-diaminopentanoyl-(2,5-di-3-aminopropyl)-glutaroyl-*N*-octadecyl-(1′-heptadecyl) amide, trifluoroacetate and 2,5-diaminopentanoyl-(2,2,5,5-tetra-3-aminopropyl)-glycyl-*N*-dioctadecyl amine, trifluoroacetate (Pfx-5); No. 6, 1:1 mixture of 2,5-diaminopentanoyl-(2,5-di-3-aminopropyl)-1,2-diaminoehtyl-*O*-octadecyl-(1′-heptadecyl)carbamic acid, trifluoride and DOPE (Pfx-7); No. 7, Bis-(2,5-diaminopentanoyl-(2,5-di-3-aminopropyl)-cystyl-*N*-dioctadecyl amine))disulfide, trifluoroacetate (Pfx-8); No. 8, lipofectin; No. 9, DC-cholesterol/DOPE 4/6.
[c]Data are expressed relative to the *N*-acyl-PE-containing liposomes, taken as 1.0, ie the numbers represent the factor by which each lipoplex is more or less toxic or active. Data from more than one series of experiments were compared using lipid No. 2 as a standard.
[d]Transfection efficiency was measured by EGFP fluorescence as in Figure 10 and corrected for total cell esterase activity as reflected in the total fluorescence of calcein blue (see Materials and methods).

represent the factor by which either of these parameters is higher in the test system. We found that the transfection activity of the *N*-C12-DOPE/DOPC (70:30) liposomes was generally in the range of that found for cationic lipoplexes under these conditions. Some lipoplexes gave considerably lower and some considerably higher activity. Lipoplexes containing 3β[*N*-(dimethylaminoethane)-carbamoyl]cholesterol and dioleoylphosphatidylethanolamine (DC-chol/DOPE) were particularly active. However, like all cationic lipoplexes, they were considerably more toxic than the liposomes to the particular cells used in these experiments, especially at the higher concentration. This could be observed in the lower calcein blue fluorescence after treatment with the lipoplexes (Table 1 data) as well as the microscopic observation of rounded and disrupted cells after treatment (data not shown). In several cases the transfection efficiency of cationic lipoplexes actually decreased relative to liposomes at the higher concentration, probably as a result of their toxicity. No toxicity was observed with the liposomally encapsulated DNA. Interestingly, treatment with the liposomes commonly caused an increase in the final calcein blue fluorescence between 10 and 30%, possibly as a result of protection from the effects of the incubation in serum-free medium.

The importance of the relatively low toxicity of this liposomal plasmid DNA delivery system is not completely apparent in the tissue culture systems because the transfection efficiency reaches saturation at the relatively low levels of DNA used in the experiments above. However, the situation *in vivo* is expected to be much different. The large excess of nonspecific binding sites *in vivo* may necessitate the use of high levels of DNA and/or multiple injections for efficient expression in the target cells. There may be a limit to the use of cationic lipoplexes in this situation because of their toxicity.

Transfection in ascites fluid

The transfection of ovarian cancer cells must also occur in the physiological millieu of the peritoneal cavity, where serum proteins extravasate from the circulation in tumor-bearing animals. The known propensity of serum proteins to interfere with liposomal delivery has led to several strategies to overcome this problem. The unique characteristics of intraperitoneal (IP) delivery include the possibility of overcoming serum protein effects by simply saturating the system with liposomes. Preliminary experiments have shown that IP injection of high concentrations (approximtely 5–10 mg of lipid, 0.5 ml volume, approximately 0.5 g lipid/kg body weight) of empty *N*-C12-DOPE/DOPC (70:30) liposomes into normal mice results in no discernible toxicity over the course of 2 weeks (not shown). Furthermore, no *in vitro* toxicity was observed at these high lipid concentrations (data not shown). Therefore, keeping in mind the apparently low toxicity of the *N*-C12-DOPE/DOPC liposomes, we tested the transfection of OVCAR-3 cells *in vitro* at a high liposome concentration in the presence of peritoneal fluid from OVCAR-3 ascites tumor-bearing mice. A cell-free lavage was taken from a SCID mouse 7 weeks after injection of OVCAR-3 cells, concentrated to an estimated 10 mg/ml of protein with a 10000 m.w. cutoff spin concen-

trator and supplemented with Ca^{2+} and Mg^{2+} to within the normal serum range. This was added to cultured OVCAR-3 cells and mixed with liposomes for transfection (see Materials and methods). Incubations and assay of enhanced green fluorescent protein (EGFP) production were as in the experiments above. We found that the presence of the IP fluid considerably decreased the total transfection efficiency as measured by EGFP production and shown in Figure 10. However, there was still significant remaining expression of EGFP, approximately 30% in this case, and inspection of the samples by fluorescence microscopy suggested that the actual percentage of transfected cells, although uneven across a tissue culture well, appeared to remain relatively high (Figure 11). Therefore, the serum-related reduction in EGFP may have been primarily due to a general reduction in the amount expressed in each cell rather than a reduction in the percentage of cells transfected. These results suggest that further optimization of this system on the scale already attempted for cationic lipoplexes may eventually lead to a gene delivery system useful for IP transfection of ovarian cancer cells.

Discussion

We have devised a novel nonviral system for delivery of plasmid DNA to living cells without the use of cationic lipids, phosphatidylethanolamine, cationic polymers or viral components. Nonviral systems that deliver and express plasmid DNA in mammalian cells must satisfy several major requirements. First, the DNA should be protected from degradation. This can be accomplished by actual encapsulation in a liposomal membrane or by complexation with a condensing agent, such as cationic lipids. However, only liposomal encapsulation protects the condensed DNA from the external environment until delivery, allowing great flexibility in the choice of methods of condensation. Secondly, the system should be flexible enough to allow various surface chemistries that may confer targeting specifically to sites of interest. A disadvantage of cationic complexes is that they would be expected to nonspecifically interact with the predominantly negatively charged surfaces of most cells. True liposomal systems would have the advantage of possible incorporation of various targeting moieties. Such systems could also potentially utilize any membrane-forming lipid components of various charges and hydrophilicities to tailor the characteristics of the liposomal composition to the particular intended physiological site of action, including possible serum protein avoidance mechanisms. The size of the resulting particle is a third consideration. Liposomes above a certain size will be cleared from circulation[14] and also may not fuse as efficiently as smaller liposomes. We have demonstrated here a method designed for encapsulation of plasmid DNA into true liposomes. The preparations contain particles predominantly of a small size (<400 nm) with characteristics strongly suggesting true encapsulation of condensed DNA. Evidence for true liposomes include electron

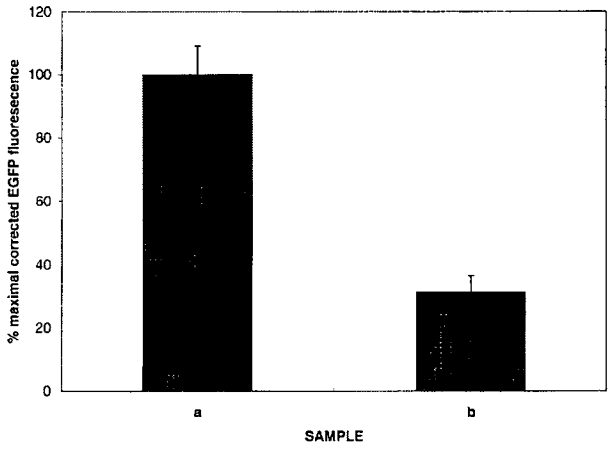

Figure 10 Transfection via N-C12-DOPE/DOPC (70:30) liposomes in mouse ascites fluid compared with buffer. Ascites was obtained from the lavage of a tumor-bearing SCID mouse as described in Materials and methods. Cells were incubated with plasmid DNA-containing liposomes (not a pellet) at a final concentration 10 mM total lipid in HBSS (a) or HBSS with ascites fluid (b) at a final protein concentration of approximately 5 mg/ml and a final concentration of 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}. After 3 h of incubation, the transfection solution was replaced with serum- and butyrate-containing medium for approximately 20 h. Expression of EGFP was measured via its fluorescence normalized to total cellular esterase activity as described in Materials and methods. An estimate of units per cellular protein is given in Materials and methods. Error bars are ± s.d.

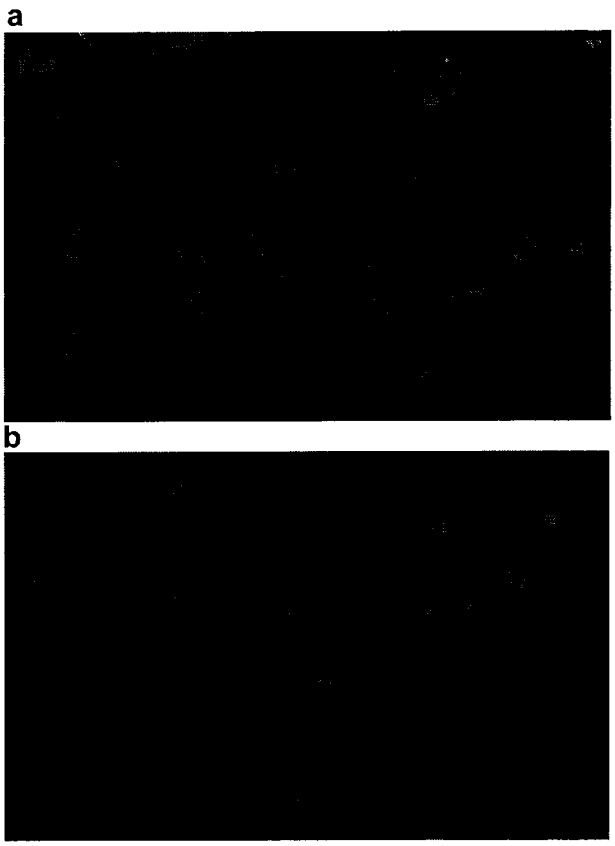

Figure 11 Fluorescent photomicrographs of OVCAR-3 cells transfected with N-C12-DOPE/DOPC (70:30) liposomes in buffer or mouse ascites fluid. Cells treated as described in the caption to Figure 10 were photographed as described in Materials and methods. (a) Represents transfection without peritoneal ascites fluid and (b) with peritoneal ascites fluid (as in Materials and methods). Cells are confluent in these views.

microscopic observations and entrapment of sucrose in lipid assemblies that also associate with transfection activity.

Certain issues are unique to liposomal systems. One is the necessity to maximize encapsulation efficiency. This can be addressed by high lipid-to-water ratios during encapsulation. More important is the efficiency of loading of the liposomes, ie the final ratio of encapsulated DNA-to-lipid. In this system, we have used the two-step method with two different sets of conditions to produce liposomes with two different size and encapsulation profiles. We have achieved a protected DNA-to-lipid ratio as high as approximately 5.6 μg/μmol for extruded liposomes thus far under one set of conditions. We have observed a pelleted DNA-to-lipid ratio as high as 20 μg/μmol for one preparation (data not shown). For the liposomes tested in the experiments presented here, the ratio was in the range of 1 μg/μmol as determined by digestion experiments. If every plasmid monomer were surrounded by a 200 nm diameter membrane, this ratio would only be about 8. Smaller liposomes would have higher ratios. Therefore, this method apparently produces a relatively high loading efficiency, notably without the use of toxic cationic lipids.

Many other methods have been developed to encapsulate water soluble materials into liposomes, some of which have also been used to encapsulate plasmid DNA, such as reverse phase evaporation[44] and dehydration-rehydration.[45] Plasmid DNA has been found to be associated with liposomes made from these methods after extensive wash. However, our data cannot be compared directly since the liposomes were not subjected to DNase digestion in the previous studies. Therefore, it is unclear whether the liposome-associated DNA was indeed encapsulated inside the liposomes or simply bound to the surface of the liposomes or both. In a later attempt to identify the best method for plasmid DNA encapsulation, Monnard *et al*[46] compared liposomes made from three methods: (1) reverse phase evaporation, (2) dehydratio–rehydration, (3) freeze–thaw. The liposomes were made under similar conditions with the three methods and were digested by DNase. All three methods showed DNA protection against DNase digestion. A relatively high percentage of protection, up to 50% of the starting linearized plasmid DNA, was obtained with the freeze–thaw method. However, the starting amount of DNA, 10 μg, was low compared with the amount of lipid, approximately 100 μmol, which inevitably would produce many empty liposomes and a low ratio of encapsulated DNA-to-lipid. The ratios in such preparations appeared to be in the range of 0.05–0.1 μg/μmol. Furthermore, a cationic lipid was necessary to produce the highest percentage of protection from DNase digestion, suggesting the possibility that some protection was the result of complex formation rather than true liposomal encapsulation.

Since condensation can reduce the size of plasmid DNA, there have been attempts to encapsulate DNA condensed with polylysine[6] or polyamines[47–49] into liposomes. Encapsulation of spermine pre-condensed linear DNA into liposomes was previously reported.[49] However, the DNA concentration was 10- to 100-fold lower than we have used. Maximal loading efficiency of these liposomes appeared to be in the range of 0.02–0.2 μg/μmol DNA/lipid. Baeza *et al*[47] and Ibáñez *et al*[48] claimed encapsulation of 1–4 μg/μmol of spermidine pre-condensed SV40 DNA based on analysis of a 200000 *g* pellet of the preparation. However, the preparations were not dialyzed into high salt buffer to remove spermidine and reverse condensation of unencapsulated DNA, which would pellet under these conditions. No DNase I digestions were performed with dialyzed preparations, free of external spermidine, to determine encapsulation. Therefore, a direct comparison with our methods may not be possible, although our preliminary results with this system showed that much less than 1 μg/μmol of DNA was encapsulated after dialysis and DNase digestion. In general, all studies showed increased DNA association with liposomes and improved transfection efficiency when DNA was condensed.

In order to improve DNA encapsulation and minimize the presence of empty liposomes, it can be helpful to work with high concentration DNA solutions. We found that pre-condensation at high DNA concentration produced large aggregates that were not suitable for encapsulation. Therefore, we have used reverse micelles to help achieve plasmid DNA condensation at high concentrations without extensive aggregation. Using this unique method, we have obtained small lipid particles that can protect DNA from extensive DNase I digestion, indicating that DNA is truly encapsulated into these particles. Cryo-electron microscopic observations strongly support the existence of liposomally encapsulated condensed DNA. To our knowledge, this may be the first observation of condensed DNA by cryo-EM. While this two-step emulsion condensation method is an important improvement in DNA encapsulation, further optimization of many aspects of the process are still possible. A number of parameters that affect the formation of the emulsion or subsequent formation of liposomes may be tested as potential future improvements.

Nevertheless, substantial transfection efficiency was obtained with the existing protocol when fusogenic phospholipids were used. Our data indicate that the apparent ability to condense plasmid DNA into liposomal membranes is important in terms of transfection efficiency. In particular, spermine-condensed DNA was dramatically more active than uncondensed DNA, even though there was less protected DNA in the uncondensed preparations. The fact that *N*-C12-DOPE/DOPC (70:30) had much better transfection efficiency than EPC can be explained by both the better encapsulation efficiency and the cation-dependent fusogenic nature of the former composition.[33]

Transfections of primary ovarian cancer cells have been previously reported using non-liposomal systems, such as retroviral[50] and cationic lipid vectors,[51] at efficiencies as high as 10–60% of the cells. This assessment depends of course on the plasmid and the sensitivity of the particular assay used and the amount of subliminal expression.[52] No studies are directly comparable with the particular cells and assay systems we have used. However, we have observed that the current liposomal formulation is as active as some lipoplex systems in transfection of OVCAR-3 cells in tissue culture with significant room for the kinds of optimization that have already been attempted for cationic lipoplexes. It is not yet known how this activity will translate *in vivo*, but it appears that the *N*-C12-DOPE/DOPC formulation is nontoxic and that it retains significant activity at a serum protein concentration relevant for the intraperitoneal cavity. The activity

of this liposomal system is also enhanced considerably in these studies by the use of nontoxic transgene activators, such as butyrate, which can reasonably become a part of the ultimate formulation. Further enhancement may be obtained by the use of targeting moieties on the liposomes and by enhancing expression efficiency of the plasmid itself.

Because of the apparently general applicability of the two-step reverse micelle method for liposomal plasmid DNA encapsulation, experiments are also underway to formulate other fusogenic lipid compositions with spermine-condensed plasmid for better transfection as well as targeting and potentially more diversified applications.

Materials and methods

Materials

N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (transesterified from egg phosphatidylcholine; designated Rh-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), egg phosphatidylcholine (PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-dodecanoyl (*N*-C12-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3β[*N*-(dimethylaminoethane)-carbamoyl]cholesterol (DC-cholesterol) and nuclease-free spermine tetrahydrochloride were from Sigma (St Louis, MO, USA). OVCAR-3 ovarian carcinoma cells were purchased from NCI-Frederick Cancer Research Laboratory (Frederick, MD, USA). The pEGFP-C1 plasmid, and *E. coli* DH5α competent cells were from Clontech Laboratories (Palo Alto, CA, USA). pZeoSVLacZ plasmid, competent cells, Pfx lipids (see Table 1) and Hanahan's SOC were from Invitrogen (San Diego, CA, USA). Hank's balanced salt solution (HBSS), RPMI 1640 medium, heat inactivated fetal bovine serum and Lipofectin (a 1:1 w:w mixture of *N*-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and DOPE) were from Gibco/BRL (Grand Island, NY, USA). DNase-free RNase and RNase-free DNase I were from Boehringer Mannheim (Mannheim, Germany). Agarose was from FMC Bioproducts (Rockland, ME, USA). Bacto agar, Bacto tryptone and yeast extract were from DIFCO Laboratories (Detroit, MI, USA). Calcein blue acetoxy methyl ester (CBAM), PicoGreen and SybrGreen I dyes were from Molecular Probes (Eugene, OR, USA).

Plasmid purification

The pZeoSVLacZ plasmid (6.5 kb, expresses β-galactosidase from the SV40 early enhancer–promoter and pEGFP-C1 (4.7 kb, expresses enhanced green fluorescent protein, EGFP, from a human cytomegalovirus immediate–early promoter) were purified from *E. coli* as described using a previously developed method.[53] The final ratio of OD at 260 nm to OD at 280 nm was greater than 1.9 for all preparations. Agarose gel electrophoresis indicated DNA in the expected size range (data not shown).

Phospholipid–DNA formulations

N-C12-DOPE/DOPC (70:30) or EPC liposomes were prepared by a two-step procedure. Most samples were prepared by diluting 200 μg of DNA into 125 μl of low salt buffer (LSB – 10 mM Tris-HCl, 1 mM NaCl, pH 7.0) and adding it to 1 ml $CHCl_3$ containing 30 μmol lipids in a 13 × 100 Pyrex tube while vortexing. The sample was immediately sonicated for 12 s in a bath sonicator (Laboratory Supplies Co., Hicksville, NY, USA) under maximum power to form an emulsion with plasmid DNA first. In a second step, a 125 μl aliquot of LSB containing the desired concentration of spermine (optimally 16–40 mM for *N*-C12-DOPE/DOPC) was added to this emulsion with vortexing and sonication as above. The *N*-C12-DOPE/DOPC (70:30) sample without spermine was prepared in the same way except that spermine was omitted from the second 125 μl aliquot.

The resulting emulsion was placed within a few minutes in a flask on a Rotovap (Bhchi Laboratoriums-Technik AG, Switzerland) to remove solvent while the vacuum was modulated with a pin valve at an initial vacuum of approximately 600–650 mm and while rotating the flask at its maximum rate. The vacuum was increased as rapidly as possible without excessive bubbling until the maximum vacuum was reached (approximately 730 mm). The flask was then evacuated for another 25 min. The film left on the flask was resuspended in 1 ml of 300 mM sucrose in LSB, and the sample was extruded five times through 0.4 μm polycarbonate membrane filters (Poretics, Livemore, CA, USA). The sample was then dialyzed against Hank's balanced salt buffer (HBSS) without Ca^{2+}/Mg^{2+} overnight at 4°C.

Spermine partitioning

Experiments designed to measure the partitioning of spermine between negatively charged phospholipids and DNA were performed with a dialysis device with three 250 μl chambers (Sialomed, MD, USA). The desired amount of spermine was diluted into LSB and placed in the center chamber, which was flanked by two 100000 m.w. cutoff dialysis membranes and contained 400 μg of pZeoLacZ plasmid DNA on one side and LSB alone or empty *N*-C12-DOPE/DOPC (70:30) liposomes, at a total lipid concentration of 30 mM on the other side. Each dialysis device was rotated on a 12 inch motorized wheel overnight (approximately 20 h). The DNA-containing chamber was then withdrawn with repeated pipetting to mix the sample, and it was placed in a 250 μl volume cuvette. The absorbance at 400 nm was used to monitor turbidity against the buffer background.

Spermine titration curves of DNA turbidity were constructed from dialyses with and without liposomes present. The approximate shift in the curve due to the presence of liposomes was used to calculate the relative binding constants for the lipids and the DNA assuming that each spermine molecule binds to four nucleotide phosphate groups or four phospholipids in simple equilibria with association constants K_{DNA} and K_{lipid}, respectively. In low salt, the dissociation constant for spermine from DNA is know to be in the μM range.[22,54] Therefore, the free concentration of spermine was taken as negligible at the mM spermine concentrations necessary for DNA aggregation in these experiments. The fractional neutralization of the DNA phosphate groups by spermine required for DNA aggregation, γ, was taken as 0.9 based on the data obtained in the absence of liposomes. This is the same value reported to be required for DNA condensation, agreeing with the previous observation that aggregation accompanies condensation at high DNA

concentrations.[22,54] Assuming [DNA – spm] = γ[DNA]$_{total}$ at the point of aggregation and [lipid – spm] = the shift in the curve, one can use the equation

$$K_{DNA}/K_{lipid} = [\gamma/(1 - \gamma)] \times [(Lipid_{total} - shift)/(shift)]$$

When the Lipid$_{total}$ is taken as the total concentration of negatively charged lipid exposed on the outside of the liposomes divided by four, the ratio of apparent equilibrium constants is 178, ie the spermine binding to DNA is much more avid than binding to the lipids. The ratio of binding constants and the first factor on the right are constants. Therefore, the last factor on the right can be used to calculate the shift in the spermine titration curve for DNA condensation for any total lipid concentration, including the higher effective concentration used in the emulsions.

In practice, we found that the optimal spermine concentration in the final emulsion before solvent removal was 8–20 mM spermine (in terms of the total aqueous volume), enough to neutralize 35–95% of the negative charge of the phospholipid as well as all of the DNA (data not shown). Above these levels aggregation of the lipid–DNA preparation occurred. The plasmid DNA is expected to be completely condensed in the 8–20 mM concentration range based on the above analysis. Most of the original spermine is expected to remain associated with the encapsulated DNA under these conditions.

Light microscopy of liposome samples

Light microscopy of plasmid aggregates was performed using 200 μg of pZeoLacZ plasmid in 125 μl LSB, mixed gently with 7 mM spermine in 125 μl LSB and incubated for 15 min at room temperature. For estimation of the size of our *N*-C12-DOPE/DOPC (70:30) samples, polystyrene beads with an average diameter of 269 ± 7 nm (Duke Scientific, Palo Alto, CA, USA) were diluted with H_2O to a concentration appropriate for microscopy and the *N*-C12-DOPE/DOPC (70:30) samples were used after extrusion and dialysis without further dilution (approximately 20 mM lipid). The samples were examined under an Olympus BH-2 fluorescence microscope (Olympus, Lake Success, NY, USA) at 1000×.

Particle analysis by light scattering

Particle size analysis was performed using a NICOMP 370 particle size analyzer (Paricle Sizing Systems, Santa Barbara, CA, USA). Samples were diluted approximately 10-fold for analysis. A Gaussian analysis was performed in the vesicle mode and number weighted averages are reported.

Freeze–fracture TEM

About 2 μl of sample was deposited between two Balzers copper double replicating holders and frozen in liquid propane. The sample was fractured at –100°C, 10^{-6}–10^{-7} barr and shadowed with platinum (∠45°) and carbon in a Balzers BAF 400 freeze–fracture device. Replicas were digested with 5% bleach overnight, washed with distilled water and mounted on 300 mesh grids. The images were obtained with a Philips (Mahwah, NJ, USA) 300 TEM.

Cryo-transmission electron microscopy

For the EPC sample and spermine-aggregated DNA, copper grids coated with a holey carbon support were used without further treatment. For *N*-C12-DOPE/DOPC (70:30) samples, EM grids with a holey carbon film were rendered positively charged by placing a drop of a 0.1 mM polylysine solution on the grid surface and allowing it to incubate for 1 min. The polylysine was blotted off and the grid rinsed with several drops of distilled water followed by several drops of sample buffer. A 5 μl aliquot of sample was then placed on the grid, blotted to a thin film and immediately plunged into liquid ethane. The grids were stored under liquid nitrogen until used. They were viewed on a Philips CM12 transmission electron microscope (Mahwah, NJ, USA) operating at an accelerating voltage of 120 kV. A 626 cryoholder (Warrendale, PA, USA) was used to maintain sample temperature between –177°C and –175°C during imaging. Electron micrographs were recorded of areas suspended over holes under low electron dose conditions. Magnifications of 35000× or 60000× and underfocus values of 1.8–2.5 μm were used.

Agarose gel analysis

A 50 μl aliquot of the desired preparation was diluted into 145 μl HBSS without Ca^{2+}/Mg^{2+} and 1 μl of 0.2 M $MgCl_2$, and 2 μl DNase I (20 units) were added with mixing. After a 6 h incubation at room temperature, 2 μl of 0.5 M EDTA was added to stop the reaction. For undigested controls, a 50 μl aliquot of each sample was mixed with 150 μl HBSS (w/o Ca^{2+}/Mg^{2+}). Samples were then extracted with phenol/$CHCl_3$/isoamyl alcohol and precipitated with ethanol as described.[55] The pellet was dissolved in 20 μl TE (pH 8.0), 5 μl of which was loaded on a 0.8% agarose gel. The gels were stained with a 1:10000 dilution of stock SYBR Green I nucleic acid gel stain (Molecular Probes) for 30 min and visualized on a Foto-Spectrum ultraviolet transilluminator (light box). Photographs were taken on the light box with a Polaroid MP 4+ camera system. These photographs were then scanned on a ScanJet IIC (Hewlett Packard, Palo Alto, CA, USA) and digitized with Aldus Photostyler (U-Lead Systems, Torrance, CA, USA).

Quantification of DNA

The PicoGreen fluorescent assay[56] was used to quantify DNA that had been extracted by the phenol/chloroform procedure.[55] A working solution was prepared by adding 100 μl PicoGreen stock (Molecular Probes) to 20 ml TE (pH 7.5). The extracted sample was first diluted 100× with TE (pH 7.5). Then, a 14 μl aliquot of the diluted sample was mixed with 986 μl TE (pH 7.5) and 1 ml PicoGreen working solution. The mixture was incubated in the dark at room temperature for 4 min. The PicoGreen fluorescence was recorded at room temperature on a PTI Alphascan fluorometer (South Brunswick, NJ, USA) with excitation wavelength of 480 nm and emission wavelength of 520 nm with a >500 nm highpass filter (Schott Glass Technologies, Duryea, PA, USA). The fluorescence of 1 ml TE (pH 7.5) and 1 ml PicoGreen working solution mixture was used as blank. The percent DNA being protected from DNase I digestion was calculated by subtracting the blank and taking the undigested sample as 100%. Under our experimental conditions, the fluorescent signal from digested DNA was insignificant (data not shown).

Cationic lipoplex preparation

Complexes of cationic lipids and helper lipids with plasmid DNA were prepared shortly before use. For Lipofec-

tin, the lipid alone was incubated in serum-free medium for approximately 45 min before complexation with DNA, as suggested by the manufacturer. Equal volumes of 4 μg/ml DNA and 40 μg/ml lipid or equal volumes of 20 μg/ml DNA and 200 μg/ml lipid, all in serum-free RPMI 1640 medium, were mixed and allowed to incubate for approximately 10–15 min before addition to wells of the tissue culture plates. Ca^{2+} and Mg^{2+} were adjusted to 1.2 mM and 0.8 mM final concentration, respectively, by addition of a concentrated stock just before addition of the lipoplexes to the cells. The ratio of lipid/DNA used for Lipofectin was based on an optimization comparing several ratios.

DC-Cholesterol/DOPE (4/6) complexes were formed essentially as previously described[57] and used within 15 min. The optimized DNA/lipid ratio was used in all experiments, ie 4 μg/ml DNA was mixed with equal volume of 20 μg/ml lipid or 20 μg/ml DNA was mixed with equal volume of 100 μg/ml lipid.

All other complexes were formed using a set of cationic lipids or lipid mixtures from a single manufacturer (Invitrogen). These were prepared as suggested by the manufacturer at the 1× concentration and at the suggested lipid/DNA ratios.

Transfection assays

OVCAR-3 cells were plated at 1×10^5 cells per ml in 24-well plates or at 2×10^5 cells per ml in 96-well plates in 1 ml or 0.1 ml per well, respectively, of RPMI 1640 with 10% heat inactivated fetal bovine serum. Cells were allowed to grow for 2 days (approximately 40–48 h) before transfections were performed. At this point the cells were at confluency. This condition was chosen better to model the tumor nodules that line the mesothelium in the actual disease, to enhance the signal by maximizing the number of cells, to decrease the tendency of wash procedures to detach cells and to minimize any artifacts that may occur from the exposure of the tissue culture plate plastic during the transfection procedure. The plates were aspirated to remove medium and washed once with Dulbecco's phosphate-buffered saline followed by aspiration.

Transfection solutions (0.5 ml per well for 24-well plates, 0.1 ml per well for 96-well plates) were prepared beforehand by dilution of dialyzed samples containing the pEGFP-C1 plasmid 10-fold into serum-free medium (approximately 2 mM total lipid) and were then added to the wells and incubated at 37°C. This dilution results in an encapsulated DNA concentration of 1–2 μg/ml and a total DNA concentration of approximately 14 μg/ml in experiments where external DNA was not removed (Figures 7, 8, 9a, 10 and 11). Where liposomal pellets were used (see below), the final DNA concentrations were approximately 1–2 μg/ml (Figure 9b) or as indicated (Table 1). In the experiments in Table 1 and Figures 10 and 11, an aliquot of a stock solution was added to adjust the Ca^{2+} and Mg^{2+} concentrations to 1.2 and 0.8 mM, respectively, just before addition to the wells. After 3 h, the samples were aspirated and medium containing 10% heat inactivated fetal bovine serum and either 5 μM trichostatin A (Figures 7 and 8) or 5 mM sodium butyrate (Figures 9–11 and Table 1) was added followed by incubation at 37°C for 18–22 h. (The latter histone deacetylase inhibitors have been shown to enhance transgene expression under the CMV promoter on a short-time scale.[41,42] We found that these inhibitors enhance the transgene expression of both cationic lipoplexes and the liposomal system in the range of two- to 10-fold at 2 μg/ml DNA after 18–22 h, similar to previous results[43].) At this point the cells were washed with Dulbecco's phosphate-buffered saline (PBS) and photomicrographs were taken (Olympus IMT-2 inverted microscope using the 10× objective). The PBS was then aspirated and 0.5 ml (0.1 ml for 96-well plates) of 5 μM calcein blue acetoxy methyl ester (CBAM) in PBS was added to each well and incubated for 40 min at room temperature. Cells were washed again with PBS, aspirated, and 0.5 ml (0.1 ml for 96-well plates) of 1% $C_{12}E_8$ in TE buffer (pH 8.0) was added to each well. After shaking for 10 min, the fluorescence of the plates was measured in a Cytofluor II fluorescent plate reader (PerSeptive Biosystems, Framingham, MA, USA).

Readings for calcein blue loaded into the live cells were made at excitation of 360 nm and emission of 460 nm with a gain of 80. These were previously verified to be linear with the number of cells originally plated up to a level where confluence was observed. Readings for EGFP were at excitation of 450 nm and emission of 530 nm with a gain of 95. Eight reads per well were averaged and each plate was read twice and the average of the two readings taken. The plates were then washed twice with the 1% $C_{12}E_8$ solution and fluorescent readings were taken again in the same solution. Untreated wells were used to correct for changes between the readings before and after the $C_{12}E_8$ washes. This correction was always less than 10%. The correction of background well by well was performed next by subtracting the readings after detergent washes from those in the original detergent solution on a well by well basis. The EGFP readings in each well were then corrected by dividing by the total calcein blue reading in each well, ie the readings are normalized for total cellular esterase activity.

An approximate conversion to EGFP fluorescence per unit cellular protein could be estimated from the data in the figures by measuring the average protein concentrations of 48 h cultures of the OVCAR-3 cells in 24- and 96-well plates extracted with 1% Triton X-100 detergent. A bicinchoninic acid assay (Pierce Chemical, Rockford, IL, USA) was used with bovine serum albumin as a standard. A few examples follow. For the the 24-well plate experiment in Figure 8, bar 'a', the total average background-corrected fluorescence reading per well was 670 units. From a separate plate, the average total cellular protein per well at the time of transfection (48 h) was approximately 88.4 μg per well giving 7.6 fluorescence units per μg of total cellular protein in a volume of 0.5 ml. In Figure 9, the data for bar 'a' (96-well experiment), represents an average background-corrected EGFP fluorescence of approximately 420 units per well with an average total cellular protein concentration of 27 μg per well, giving 15.5 fluorescence units per μg of total cellular protein in a total volume of 0.1 ml. In Figure 10 (96-well experiment), the bar 'a' fluorescence reading was 103 fluorescence units per μg of cellular protein.

To model intraperitoneal delivery (data in Figures 10 and 11), the transfection was performed by first adding 50 μl of a concentrated cell-free lavage fluid from the peritoneal cavity of tumor-bearing SCID mice (see below) to each aspirated well of a 96-well plate with OVCAR-3 cells grown as described above. To this was added 50 μl of *N*-

C12-DOPE/DOPC liposomal DNA prepared as described above and resulting in a final lipid concentration of approximately 10 mM and a final encapsulated DNA concentration of approximately 7–14 μg/ml (total DNA of 67 μg/ml). Incubations were performed as described above. In this case, the peritoneal lavage fluid was adjusted to approximately serum levels of Ca^{2+} and Mg^{2+} (1.2 mM and 0.8 mM, respectively) by adding a concentrated stock. The liposomal–DNA solution was also adjusted to the same levels of Ca^{2+} and Mg^{2+} by addition of the concentrated stock just before addition of the liposomes to the cells.

Sedimentation of plasmid DNA and lipid particles

Liposomes were prepared by sedimentation to remove external DNA for the experiments shown in Figure 9. *N*-C12-DOPE/DOPC (70:30) liposomes were prepared by the two-step method with spermine, except that 200 mM sucrose was included in the LSB used to form the emulsion. Headgroup labeled lissamine rhodamine B-phosphatidylethanolamine (Rh-PE) was also added as a lipid probe at 10 μg/ml. A 500 μl aliquot of the preparation was then centrifuged at 16000 *g* for 3 h. The pellet was resuspended in HBSS without Ca^{2+}/Mg^{2+}, centrifuged at 16000 *g* for 3 h, and resuspended again in 500 μl of the same buffer. Aliquots of 50 μl of each fraction were taken for DNase I digestion as described above. After phenol/$CHCl_3$ extraction and ethanol precipitation, the plasmid DNA in each aliquot was measured by PicoGreen assay as described above and used to calculate the percentage of plasmid DNA protected and the total plasmid DNA in each fraction.

To measure the distribution of lipids, a 40 μl aliquot of each fraction was dissolved in 0.2% $C_{12}E_8$ in a total volume of 2 ml and the fluorescence monitored with an excitation wavelength of 560 nm with a 550 ± 20 nm bandpass filter (Melles Griot, Irvine, CA, USA) and an emission wavelength of 590 nm.

As a control, empty *N*-C12-DOPE/DOPC (70:30) liposomes were prepared as above. After dialysis, 100 μg of EGFP plasmid was added to 500 μl of the sample. The sample was then centrifuged and quantified for lipids and plasmid DNA as described above.

Lavage fluid

A cell-free 6 ml HBSS lavage was taken from a SCID mouse 7 weeks after injection of OVCAR-3 cells and was concentrated to 0.5 ml with a 10000 m.w. cutoff spin concentrator. Protein recovery is approximately 60%. This fluid comprising approximately 10 mg/ml protein in HBSS was supplemented with Ca^{2+} and Mg^{2+} to within the normal serum range (1.2 mM and 0.8 mM, respectively), added to cultured OVCAR-3 cells and gently mixed in equal volume with liposomes in HBSS at the same Ca^{2+}/Mg^{2+} level to give a final lipid concentration of 10 mM.

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